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Schipper, Lidewij; van Dijk, Gertjan; van der Beek, Eline M.

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Milk lipid composition and structure; The relevance for infant brain development☆

Lidewij Schipper[1,2,*], Gertjan van Dijk[2] and Eline M.van der Beek[1,3]

[1] Danone Nutricia Research, Utrecht, The Netherlands
[2] GELIFES, Groningen Institute for Evolutionary Life Sciences, University of Groningen, Groningen, The Netherlands
[3] Department of Pediatrics, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

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Abstract – The neurocognitive development of infants can be positively associated with breastfeeding exclusivity and duration. Differences in dietary lipid quality between human milk and infant milk formula may contribute to this effect. In this review, we describe some of the known differences between human milk and infant milk formula in lipid quality, including fatty acid composition, complex lipids in the milk fat globule membrane as well as the physical properties of lipids and lipid globules. We describe some of the underlying mechanism by which these aspects of lipid quality are thought to modulate infant brain development such as differences in the supply and/or the bioavailability of lipids, lipid bound components and peripheral organ derived neurodevelopmental signals to the infant brain after ingestion and on longer term.

Keywords: human milk / infant milk formula / lipid composition / lipid structure / infant brain development

Résumé – Composition et structure des lipides du lait ; importance pour le développement du cerveau du nourrisson. Le développement neurocognitif des nourrissons peut être associé de façon positive à l'exclusivité et à la durée de l'allaitement. Les différences qualitatives des lipides alimentaires entre le lait humain et les préparations lactées pour nourrissons pourraient contribuer à cet effet. Dans la présente étude, nous décrivons certaines des différences connues entre le lait humain et les préparations pour nourrissons en ce qui concerne la qualité des lipides, y compris la composition en acides gras, les lipides complexes de la membrane des globules gras du lait ainsi que les propriétés physiques des lipides et des globules lipidiques. Nous décrivons certains des mécanismes sous-jacents par lesquels ces aspects qualitatifs des lipides pourraient moduler le développement du cerveau du nourrisson, comme les différences dans l'apport et/ou la biodisponibilité des lipides, des composants liés aux lipides et des signaux de neuro-développement issus d'organes périphériques et allant au cerveau du nourrisson juste après l'ingestion et à plus long terme.

Mots clés : lait maternel / préparation lactée pour nourrisson / composition lipidique / structure lipidique / développement du cerveau du nourrisson

1 Infant feeding mode and neurocognitive development

For mammals, the first and only source of nutrition directly after birth is maternal milk. For humans, the current recommendations by the World Health Organization (WHO) for infant feeding are to exclusively breastfeed infants up to at least 6 months of age, with continued breastfeeding along with appropriate complementary foods up to 2 years of age or beyond. Although most mothers start breastfeeding, many children do not receive mothers own milk exclusively for 6 months (World Health Organization (WHO), 2017). When mothers do not breastfeed for medical or other reasons, Infant Milk Formula (IMF) should provide an adequate alternative. Whereas human milk and IMF may be similar in energy content and macronutrient composition that are needed for infant growth and development, breast fed infants appear to have several advantages over formula fed infants when it

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*Correspondence: lidewij.schipper@danone.com

comes to (long-term) health outcomes including immune, gut and metabolic health (Harder *et al.*, 2005; Jackson and Nazar, 2006; Guaraldi and Salvatori, 2012). Regarding brain development, there are several reports that show that breastfeeding exclusivity and duration can be positively associated with *e.g.* brain structural development (Deoni *et al.*, 2013, 2018; Herba *et al.*, 2013; Kafouri *et al.*, 2013), cognitive function (Anderson *et al.*, 1999; Belfort *et al.*, 2013; Leventakou *et al.*, 2015), behaviour, school performance (Heikkila *et al.*, 2014) and food intake regulation (Li *et al.*, 2010; DiSantis *et al.*, 2011). This suggests that factors beyond bulk nutritional constituents contribute to the differences observed between breast- and formula-fed infants. Indeed, brain development and function can be influenced by the behavioral aspects of infant feeding (*e.g.* breast *vs* bottle feeding (Li *et al.*, 2010; DiSantis *et al.*, 2011), feeding on demand *vs* fixed schedule (Iacovou and Sevilla, 2013), self-regulation *vs* parental encouragement for bottle emptying (Li *et al.*, 2008), mother-child bonding (Britton *et al.*, 2006)), as well as compositional differences between human milk and formula such as the presence of bioactive components in human milk including hormones, growth factors and immune factors (Savino *et al.*, 2009; Field, 2005; Ballard and Morrow, 2013; Grey *et al.*, 2013). Furthermore, there are specific differences between human milk and IMF in the quality of nutrients, especially with regard to the quality of the lipid fraction. For instance, total lipid content in IMF is fixed whereas in human milk it is variable between and within individuals, changing over the course of lactation, during the day and even during a single feed (Saarela *et al.*, 2005; Kent *et al.*, 2006). In addition, the quality of the lipid fraction in human milk differs from that in IMF in at least two more aspects. First, the composition of individual fat components, specifically the types of fatty acids and bioactive lipid ingredients that make up the total fat content of milk, is not the same. Second, the (supra)molecular structure in which lipids are organized is substantially different between human milk and IMF. These specific aspects of lipid quality and how they may relate to infant brain development and function will be addressed in more detail below.

2 Milk fatty acid composition

Approximately 98% of the lipid fraction in human milk consists of triglycerides, each containing three fatty acids (FA) (Hamosh *et al.*, 1985; Jensen *et al.*, 1990). The majority of FA in human milk are saturated fatty acids (SFA) (German and Dillard, 2010), followed by mono-unsaturated fatty acids (MUFA) and about 20% of the FA in human milk are omega (n)-3 or n-6 polyunsaturated fatty acids (PUFA). These include longer chain PUFA (carbon chain-length >20, LCPUFA) of the n-6 and n-3 family, such as arachidonic acid (C20:4 n-6; ARA) and docosahexanoic acid (C22:6 n-3; DHA), as well as their respective C18 precursors linoleic acid (C18:2 n-6; LA) and alpha-linolenic acid (C18:3 n-3; ALA) that can be converted to LCPUFA after ingestion (Salem *et al.*, 1996).

Whereas the proportion of SFA and MUFA in (mature) human milk are relatively constant, the (LC)PUFA profile may vary in relation to maternal dietary fatty acid intake and fatty acids released from maternal adipose tissue stores or the liver (Makrides *et al.*, 1995; Innis, 2007). In particular, maternal dietary intake of ALA, LA and DHA affects the amounts of these specific PUFA in human milk (Koletzko *et al.*, 1992; Yuhas *et al.*, 2006). For instance, the marked increase in dietary intake of LA in the past 100 years, seen in Western-industrialized societies as a result of increased use of vegetable oils rich in LA (Kris-Etherton *et al.*, 2000; Sanders, 2000; Wolmarans, 2009), is also reflected in higher LA content in milk of women from Europe, Australia and Northern America (Ailhaud *et al.*, 2006; Gibson *et al.*, 2011). Whereas the recommendations for fatty acid composition in IMF have been based on human milk (Efsa panel, 2014), fluctuations from day to day and over the course of lactation would be difficult to mimic. Moreover, the recommended PUFA composition for IMF has been based on milk samples analysed in a small set of Caucasian women in a Western industrialized society context (Efsa panel, 2014). A debate is ongoing whether or not the FA composition in milk from this reference group can be considered the healthiest composition for infant growth and development (Kuipers *et al.*, 2005; Ailhaud *et al.*, 2006).

3 Fatty acids in the brain

Dietary supply of PUFA is essential for infant brain development and function. Nearly 60% of the dry-weight of the human brain consists of lipids (O'Brien and Sampson, 1965) and about 35% of the lipids in the grey matter are LCPUFA (Benatti *et al.*, 2004). In the neuronal membrane, the LCPUFA support physical and functional membrane properties. Longer chain LCPUFA increase membrane fluidity due to their more spacious structure and influence membrane receptor, enzyme activities and neuronal plasticity (Youdim *et al.*, 2000). The most abundant LCPUFA in the neuronal membrane are ARA and DHA, which rapidly accumulate in the human brain during the first 1000 days, supporting the rapid increase in brain volume (Martinez and Mougan, 1998). DHA and ARA contribute to membrane physical properties and have their own unique roles in brain development and function. Sufficient presence of particularly DHA in the neuronal membranes is critical as DHA contributes positively to various processes important for neuronal growth and development including modulating neural metabolism, differentiation, plasticity, neuroprotection and anti-inflammatory effects (see *e.g.* (Dyall, 2015) for review). ARA is the precursor for specific membrane derived eicosanoids which are important for immunity and immune responses including the regulation of neuro-inflammation (Duncan and Bazinet, 2010; Hadley *et al.*, 2016). Other n-3 LCPUFA in the brain, though present in much lower concentration than DHA, include eicosapentaenoic acid (EPA; C20:5n-3) and docosapentaenoic acid (n-3 DPA, 22:5n-3), that also generate lipid derived mediators that play a role in the inflammatory response (Dyall, 2015) and in particular EPA stimulates neurite outgrowth during development (Robson *et al.*, 2010). DPA from the n-6 family (n-6 DPA; 22:5n-6) is the structural homologue of DHA and typically accumulates in the brain when DHA supply is insufficient (Foot *et al.*, 1982; Carrie *et al.*, 2000). This compensatory mechanism ensures that the total brain volume remains the same, however, n-6 DPA cannot fulfill the specific neurodevelopmental roles of DHA

(Novak *et al.*, 2008; Cao *et al.*, 2009; Robson *et al.*, 2010; Katakura *et al.*, 2013). High levels of n-6 DPA are therefore considered disadvantageous. Preferential accumulation of DHA in the brain occurs at high rate between the 3rd trimester up to 2 years of age, and accumulation extends into childhood and adolescence (Martinez, 1992; Carver *et al.*, 2001). This pattern parallels active brain development and further illustrates the importance of DHA for brain development and function throughout life. Indeed, it is well-documented in animal studies that perinatal depletion of brain DHA leads to enduring abnormalities including altered morphology, neurotransmission and (long term) functional impairments including impairments in cognitive performance and visual acuity and increased risk for depression/anxiety (Neuringer *et al.*, 1986; Greiner *et al.*, 1999; Moriguchi *et al.*, 2000; Ahmad *et al.*, 2002; Carrie *et al.*, 2002; Garcia-Calatayud *et al.*, 2005; Chalon, 2006; Clouard *et al.*, 2015). Importantly, once established, these alterations cannot be fully restored by supplementing with n-3 LCPUFA during later life stages (Carrie *et al.*, 2000; Ikemoto *et al.*, 2001; Anderson *et al.*, 2005). In humans, a direct association between brain LCPUFA composition and behaviour or cognition is difficult to demonstrate as this would require the analysis of brain tissue. However, prospective studies have shown that fœtal cord blood DHA levels – which may serve as a proxy for brain PUFA status at birth – are inversely related to internalizing problem behaviour, hyperactivity and inattention in childhood (Krabbendam *et al.*, 2007; Kohlboeck *et al.*, 2011), whereas higher levels of n-3 LCPUFA are associated with better motor performance, (Bakker *et al.*, 2009), higher scores in cognitive functioning (Bakker *et al.*, 2003; Boucher *et al.*, 2011) and better neurological scores (Escolano-Margarit *et al.*, 2011). Moreover, low DHA and n-3 LCPUFA or increased n-6 LCPUFA concentration in adult brain has been related to neuropsychiatric disease (McNamara *et al.*, 2007; Conklin *et al.*, 2010; Hamazaki *et al.*, 2013). These studies show that DHA in the brain is critical for brain function and mental health in humans, further highlighting the importance of sufficient DHA accumulation during infancy.

4 Dietary fatty acids are used as building blocks for the developing brain

The developing brain relies on the plasma pool of LCPUFA for DHA accumulation. Although the brain is able to generate some DHA by endogenous synthesis from precursor n-3 LCPUFA in glial cells (Williard *et al.*, 2001), DHA synthesis in the brain occurs at a much lower rate than the total rate of brain accretion of DHA needed to support growth and development (Demar *et al.*, 2005; Kitson *et al.*, 2016). In plasma, DHA and other LCPUFA are present in two major pools;

- bound albumin, as non-esterified fatty acid (NEFA)-DHA or lysophosphatidylcholine-esterified (LPC)-DHA;
- in lipoproteins esterified to *e.g.* TG, PL, cholesterol, PL or other lysophospholipids.

There is no consensus on what lipid form in the plasma pool is preferentially used for accumulation in the brain (Bazinet and Laye, 2014; Chen *et al.*, 2015; Liu *et al.*, 2015), nor are the mechanisms agreed upon by which these various forms of circulating PUFA are delivered – either by protein-mediated and/or passive diffusion (Mitchell and Hatch, 2011). The uptake process from plasma to tissue appears to be non-selective for n-6 and n-3 LCPUFA. The accumulation of DHA in the brain is therefore dependent on the total and relative levels of DHA and other n-3 as well as n-6 LCPUFA in the plasma (Hibbeln *et al.*, 2006; Igarashi *et al.*, 2009).

In turn, the level of circulating n-3 and n-6 LCPUFA are influenced by the infant's diet. Preformed LCPUFA are present in human milk or IMF and LCPUFA can be synthesized endogenously based on the supply of the precursors LA and ALA in milk (Gibson *et al.*, 2011). The capacity of LCPUFA synthesis from LA and ALA is low in humans (Burdge and Wootton, 2002; Brenna *et al.*, 2009). Although infants (both preterm and term) may have a similar capacity as adults to convert LA to AA, and ALA to DHA (Innis, 2003, 2007), the activity seems insufficient to fulfill the high LCPUFA requirements needed for growth and development in infancy, in particular for DHA (Decsi and Koletzko, 1995; Giovannini *et al.*, 1995). Preformed DHA in the infant's diet therefore is considered conditionally essential (Muskiet *et al.*, 2004; Brenna and Carlson, 2014). Human milk contains a considerable amount of LCPUFA in preformed state. It has been suggested that one of the reasons for the advantages of breastfeeding over formula feeding regarding brain development is related to the high concentration of LCPUFA in human milk, especially the natural presence of DHA (Michaelsen *et al.*, 2009). Postmortem studies in the past have revealed that infants fed formula without DHA had lower brain DHA levels and higher n-6 LCPUFA compared to age-matched breast-fed infants (Farquharson *et al.*, 1992; Makrides *et al.*, 1994; Martinez and Mougan, 1998; Jamieson *et al.*, 1999). DHA accumulation in the infant brain is, however, not only dependent on the supply of preformed DHA in the infant diet. The biosynthesis of LCPUFA from LA and ALA in the liver and other tissues involves a series of elongation and desaturation steps. LA and ALA use the same set of enzymes for conversion to ARA and DHA and therefore compete with each other. High dietary supply of LA inhibits endogenous DHA synthesis and results in higher n-6 LCPUFA in the circulation, which further limits DHA incorporation in the developing brain as circulating n-3 and n-6 LCPUFA compete for uptake by the brain as well (Lefkowitz *et al.*, 2005; Gibson *et al.*, 2011). In line with this, the level of LA in human milk, independent of milk n-3 LCPUFA level, has been inversely correlated to infant cognitive development and function (Lassek and Gaulin, 2014; Bernard *et al.*, 2015, 2017). In a piglet study, it was found that a formula containing high LA increased the accumulation of n-6 LCPUFA and reduced DHA and other n-3 LCPUFA in the brain. Moreover, increasing DHA content failed to prevent some of the impairments in the brain fatty acid profile that were induced by the high supply of LA (Novak *et al.*, 2008). Rodent studies have shown that lowering the postnatal dietary LA supply can stimulate brain DHA accumulation (Schipper *et al.*, 2016a), alter the structural development of neuronal networks (Schipper *et al.*, 2013) and protect the developing brain against early life stress-induced neurocognitive impairments in adulthood (Yam *et al.*, 2019). Due to the persistent changes in, for example, serotonergic and dopaminergic neurotransmitter systems that are linked to early life impaired brain DHA accumulation (Chalon, 2006), it has

been hypothesized that the contemporary increase in nutritional supply of LA and low supply of DHA may also contribute to the increased incidence of neurological and psychiatric disorders such as depression and schizophrenia that has been observed over the last decades (Klerman and Weissman, 1989; Muskiet, 2010; McNamara, 2013; Grosso *et al.*, 2014).

As can be derived from the examples described above, ensuring adequate levels of n-3 LCPUFA, as well as prevention of excessive n-6 (LC)PUFA in the infant's diet appear to support n-3LCPUFA accumulation in the brain and may thereby contribute to optimal development and functioning of the infant brain. Many studies have therefore attempted to increase infant neurocognitive outcomes by (maternal) n-3 LCPUFA supplementation. However, for healthy term infants this supplementation has not always shown clear benefits (Simmer *et al.*, 2008; Delgado-Noguera *et al.*, 2010; Qawasmi *et al.*, 2012), although maternal n-3 LCPUFA supplementation during lactation seems more effective than n-3 LCPUFA supplementation of IMF (Lauritzen *et al.*, 2016). Yet, it seems unlikely that differences between LCPUFA composition in human milk and formula alone could explain the beneficial effects of breastfeeding over formula feeding on infant health and neurodevelopmental outcomes.

5 Milk fat globules

Mammalian milk has a distinct lipid composition and physical structure as a result of the physiological process by which the fat globules are produced and secreted from the mammary gland. Human milk lipid droplets are large, ranging in diameter from 0.2 to more than 15 μm with an average mode diameter of 4 μm in mature milk (Michalski *et al.*, 2005). Lipid globules are larger in early colostrum than in mature milk (Simonin *et al.*, 1984; Michalski *et al.*, 2005; Zou *et al.*, 2012), but the size remains stable during the day (Michalski *et al.*, 2005) and during a single feed (Zou *et al.*, 2012). In mammary gland cells, milk triglycerides are synthesized in the endoplasmatic reticulum and aggregate in small lipid droplets that are surrounded by a single layer of polar lipids and proteins. In the cytoplasm of the mammary gland cell, several of these microlipid droplets fuse to form larger lipid droplets that migrate through the cell to their secretion site. Upon secretion, the plasma membrane of the mammary gland cell encloses the lipid droplet, resulting in a three-layer biological membrane that surrounds the large TG core, which is known as the milk fat globule membrane (MFGM) (Wooding, 1971; Mather and Keenan, 1998; Heid and Keenan, 2005; Martini *et al.*, 2016). The composition of the MFGM equals that of other biological membranes, containing complex polar lipids such as phospholipids (PL), gangliosides, sphingomyelin and cholesterol (Lopez and Menard, 2011), which together represent the remaining 1–2% of the total lipid fraction in milk (Singh, 2006; Contarini and Povolo, 2013). The PLs are mainly located in the outer bilayer of the MFGM, while cholesterol and sphingomyelin are primarily aggregated in rigid domains in the membranes called lipid rafts (Gallier *et al.*, 2010). The most important PLs in the MFGM are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS) and sphingomyelin (Contarini and Povolo, 2013; Giuffrida *et al.*, 2013).

Whereas factory-produced composition of IMF is based on the total lipid content and fatty acid composition of human milk, it does not take into account the specific structural organization of lipid droplets seen in human milk. Due to processing steps like homogenization and emulsification in IMF production, the lipid droplets have a much smaller diameter (about 0.3–1.0 μm) compared to those seen in raw milk (Michalski *et al.*, 2005; Ye *et al.*, 2008). Moreover, there is no biological membrane surrounding the lipid globules in standard IMF. Most commercially available infant formulas contain only triglycerides as source of lipids although IMF's with added MFGM fragments are increasing in popularity.

6 Milk fat globule membrane (components) as bioactive ingredient

Although representing only a small proportion of the total lipid fraction, the natural presence of MFGM complex lipids in human milk may contribute to the differences in neurocognitive development observed between breast- and formula fed infants. Studies in rodents and piglets have shown that early life dietary supplementation with MFGM or individual MGFM components such as gangliosides and sphingomyelin can positively influence brain development and functions including neuroplasticity (Guillermo *et al.*, 2015), myelination (Oshida *et al.*, 2003) and cognitive performance (Wang *et al.*, 2007; Vickers *et al.*, 2009; Liu *et al.*, 2014a; Guan *et al.*, 2015). Interestingly, a recent study in growth-restricted rat pups suggested that supplementation with MFGM fragments resulted in better neurodevelopmental outcomes than supplementation with some of the individual components present in MFGM (Brink *et al.*, 2019). Human infants born preterm showed improved neurobehavioral development during infancy when exposed to sphingomyelin-fortified milk (Tanaka *et al.*, 2013) and healthy term infants showed improved cognitive function at 6 months of age following dietary ganglioside supplementation (Gurnida *et al.*, 2012). Moreover, at 12 months of age, the cognitive score of infants exposed to an IMF with reduced protein density and added MFGM fragments until 6 months of age was higher than that of infants fed standard formula and closer to that of a breast fed reference group (Timby *et al.*, 2014). It is hypothesized that the complex lipids in the MFGM may serve as building blocks and or developmental signals for the infant brain. Like LCPUFA, complex lipids in MFGM such as PL, sphingolipids, gangliosides, and cholesterol can also be found as structural components of neuronal membranes (Kracun *et al.*, 1992; Bjorkhem and Meaney, 2004; Posse de Chaves and Sipione, 2010; Zhang and Liu, 2015). These lipids play an important role in brain development, affecting a variety of processes including neurotransmission, neurogenesis, synaptogenesis, modulating synaptic transmission, cell proliferation, and neuronal differentiation and myelination (Mauch *et al.*, 2001; Saher *et al.*, 2005; Palmano *et al.*, 2015). Moreover, some of these complex lipids in MFGM contain (conditionally) essential nutrients required for brain development such as sialic acid, a component in gangliosides, and choline, present in phosphatidylcholine and sphingomyelin (Zeisel, 2000, 2004; Wang, 2012). These complex lipids may be absorbed by the intestine as whole (McJarrow *et al.*, 2009) or as individual

essential components after digestion, and transported to the brain where they are incorporated in neuronal membranes or used again as a precursor for de novo biosynthesis of brain lipids (Reis *et al.*, 2016). A higher dietary supply of MFGM (components) therefore increase the presence of these lipids in the neuronal membranes. Indeed, breast-fed infants were shown to have a higher brain ganglioside and sialic acid concentration than infants that had been fed a standard formula in which the content of these components is low (Wang *et al.*, 2003). In rats, early life supplementation with dietary gangliosides increased brain ganglioside content (Park *et al.*, 2005; Gustavsson *et al.*, 2010) and the brain phospholipid composition of rats artificially reared on a formula supplemented with MFGM, compared to standard formula, was closer to that of mother-reared rats (Moukarzel *et al.*, 2018). Although there is no information available on dietary cholesterol and brain cholesterol levels in human infants, increased brain cholesterol levels were found after early life dietary supply of cholesterol in piglets (Boleman *et al.*, 1998) and rodents (Morris and Chaikoff, 1961).

7 The physical properties of lipids and lipid globules

In addition to the different supply of bioactive lipid ingredients, the unique physical properties of lipids in human milk compared to that of lipids in infant formula may contribute to the beneficial effects of breastfeeding on infant brain development (Ortega-Anaya and Jimenez-Flores, 2019). The MFGM that surrounds the large lipid globule in human milk comprises a triple PL layer, each PL containing two FA tails. In human milk, about 15% of the LCPUFA are PL bound (Koletzko and Rodriguez-Palmero, 1999) while standard IMF usually contains TG only. Similar to total fat and FA composition of the TG fraction in human milk, the FA composition of the PL fraction in the MFGM is influenced by the maternal diet and the stage of lactation (Lopez *et al.*, 2008; Zou *et al.*, 2012). In itself, the molecular structure (*i.e.* PL *vs* TG) of dietary LCPUFA can influence the digestion and absorption kinetics after ingestion, and the subsequent bioavailability of LCPUFA in the plasma for the developing brain (Amate *et al.*, 2001; Michalski *et al.*, 2013). Studies in infants and healthy adult volunteers have shown that PL-bound LCPUFA in the diet, including DHA, are better absorbed than TG-bound LCPUFA (Carnielli *et al.*, 1998; Morgan *et al.*, 1998; Ulven *et al.*, 2011; Ramprasath *et al.*, 2013). The molecular structure of dietary PUFA may differentially affect their distribution in plasma fatty acid pools (NEFA, LPC, esterified) after digestion and absorption and can thereby influence the total and temporal availability for uptake by the brain (Amate *et al.*, 2001; Kitson *et al.*, 2016). Regardless of the *plasma* lipid pool, there are several preclinical studies that show that dietary PL-bound LCPUFA, including DHA, more effectively target the brain than dietary LCPUFA supplied as TG (Mathews *et al.*, 2002; Wijendran *et al.*, 2002; Graf *et al.*, 2010; Liu *et al.*, 2014b; Kitson *et al.*, 2016). This also applies to the precursors of LCPUFA, as PL bound ALA was more effective than TG bound ALA in restoring brain DHA levels after perinatal dietary n-3 LCPUFA deficiency in rats (Delplanque *et al.*, 2013).

Adding to molecular structure, the *supra*molecular structure of dietary lipids in human milk may also influence the bioavailability of PUFA for the developing brain. In human milk, lipid droplets are large and are enveloped by the MFGM, whereas lipid droplets in IMF (regardless of added MFGM ingredients) are small and do not have the complex surface area. The lipid droplet size and complexity of the surface area are factors known to affect (temporal) absorption and digestion kinetics, influencing the pattern of lipid appearance in the circulation after ingestion and thus their bioavailability for developing organs (Armand *et al.*, 1996, 1999; Michalski *et al.*, 2006, 2013; Bourlieu *et al.*, 2015; van Aken, 2010). Recently, a novel concept IMF was developed with large lipid globules surrounded by a phospholipid membrane, resembling more closely the supramolecular structure of lipid droplets in human milk (Gallier *et al.*, 2015). In adult volunteers, it was shown that consumption of this concept IMF resulted in a faster postprandial increase in plasma TG and earlier peak NEFA concentrations compared to regular IMF (Baumgartner *et al.*, 2017). Whereas potential beneficial effects of this concept IMF on brain LCPUFA accumulation pattern early in life remain to be confirmed, it was shown that early life consumption of this concept IMF improved cognitive functions in mice during adolescence and adulthood (Schipper *et al.*, 2016b).

Alternatively, it can be hypothesized that the supramolecular structure of lipid droplets can influence neurocognitive function *via* the different release gut hormones that target the brain beyond satiety regulation. Due to the changes in lipid digestion and absorption kinetics, the postprandial pattern of satiety hormones is altered (Ohlsson *et al.*, 2014). The concept IMF that mimics the supramolecular structure of lipid droplets in human milk was also reported to evoke prolonged release of the small intestine derived satiety hormone cholecystokinin (CCK) (Baumgartner *et al.*, 2017). CCK and other lipid-induced satiety signals have been reported to facilitate learning and memory processes (Monnikes *et al.*, 1997; Gulpinar and Yegen, 2004; Campolongo *et al.*, 2009). A different pattern of release during critical periods of growth and development may alter developmental trajectories of brain centres involved in neurocognitive function. Studies that compare the postprandial hormone release in infants following consumption of human milk *vs* IMF are limited in number but do confirm differences depending of feeding mode (Lucas *et al.*, 1981; Salmenpera *et al.*, 1988; Slupsky *et al.*, 2017). Recent studies also showed that the profiles of fasting state appetite regulating hormones including insulin and leptin were different between breast- and formula-fed infants (Breij *et al.*, 2017; Vasquez-Garibay *et al.*, 2019). Moreover, these hormones have associated with brain development and cognitive function (Plagemann *et al.*, 2005; Farr *et al.*, 2015; Cato and Hershey, 2016).

8 Influence of other organs and organ derived factors

On the longer term, differences between breast- and formula-fed infants in growth patterns and associated differences in organ development and functions that are mediated by differences in dietary lipid quality, may also influence brain development and or function. Recent work suggests for

instance that skeletal development is a determinant of brain development, neuronal structure and behavioural function. Bone tissue secretes the hormone osteocalcin (OCN), which was shown to be higher in serum from breast- compared to formula-fed infants (Michaelsen *et al.*, 1992). OCN crosses the blood brain barrier to promote synthesis of several neurotransmitters including serotonin, dopamine, NA and reduces GABA during postnatal life (Oury *et al.*, 2013). Mice lacking OCN show increased anxiety, depression and impaired learning and memory (Oury *et al.*, 2013). OCN synthesis is dependent on the maturity of the osteoblast and on the lipid soluble factors vitamin D and K (Skjodt *et al.*, 1985; Gundberg *et al.*, 2012; van Driel and van Leeuwen, 2014), of which absorption could be increased by the supramolecular structure in human milk (Bezelgues *et al.*, 2009). Also, the generally accepted link between early life dietary supplementation with n-3 LCPUFA (and or dietary low n-6 LA exposure) and cognitive and behavioural function could be mediated, in part, by enhanced bone development. High levels of ARA-derived prostaglandin 2 were shown to impair bone formation (Baylink *et al.*, 1993) and omega-3 fatty acid supplementation in young rats and mice amplified bone formation and OCN secretion (Watkins *et al.*, 2000; Bonnet and Ferrari, 2011; Bonnet and Ferrari, 2015).

9 Implications for human health

Human infants in our modern society may face many challenges during the first 1000 days that can affect brain development and are associated with higher risks for both mental and metabolic diseases. The WHO estimates that about one-third of the adult population suffers from a mental or neurological disorder (Kessler *et al.*, 2009) and children and adolescents are increasingly affected (Polanczyk *et al.*, 2015). As effective treatment of mental disorders is not only time consuming and expensive, but often simply not possible, prevention is key. A preventative rather than reactive approach in management of brain disorders is crucial. Prolonged breastfeeding is associated with better neurodevelopmental outcomes (Horwood and Fergusson, 1998; Belfort *et al.*, 2013; Heikkila *et al.*, 2014; Horta *et al.*, 2015; Leventakou *et al.*, 2015), which extends into later in life higher educational/academic performance (Horwood and Fergusson, 1998; Victora *et al.*, 2015) and reduced risk of later mental and behavioural problems (Montgomery *et al.*, 2006; Oddy *et al.*, 2010; Hayatbakhsh *et al.*, 2012). Whereas possible confounding circumstances such as differences in parental education, health and food habits cannot be fully excluded in these human trials, the published evidence so far suggests that differences in dietary lipids quality could contribute to this.

Providing a more optimal balance of n-3 and n-6 PUFA in early life could be instrumental. Practically, this goal can be reached through more specific dietary advice to pregnant and lactating women regarding fatty acid intake. Although the importance of (maternal) dietary intake n-3 LCPUFA for infant brain development is well known to the public, the potential adverse effects of high intakes of LA, and therefore the importance of lowering levels of LA in the (maternal) diet are not yet taken into account in current dietary recommendations. Lowering dietary intake of LA can be reached by replacing the consumption of standard vegetable oils (sunflower, soybean) and food items containing substantial LA quantities such as salad dressings and snack food by alternative food products containing oils and food products lower in LA (MacIntosh *et al.*, 2013; Wood *et al.*, 2013, 2014). These dietary advices are relatively simple, cheap and home-based and therefore have the potential to reach a broad public. In line with this, the regulation for fatty acid composition in nutritional products for infants and young children should be critically reviewed. For instance, the recommendations of the 2014 European Food Safety Authority Panel on Dietetic Products, Nutrition and Allergies include for LA a lower and upper bound range of 0.5 and 1.2 g/100 kcal IMF, combined with 0.05 to 0.1 g/100 kcal IMF for ALA, an no maximal permitted LA/ALA ratio (Efsa panel, 2014). Whereas the current recommendation now include addition of preformed DHA, but not ARA, reconsideration of the upper levels of LA in particular may be a strategy that could support healthier (brain) development of infants (Kuipers *et al.*, 2005; Muskiet *et al.*, 2006; Gibson *et al.*, 2011). In addition to lipid composition, the supramolecular structure of dietary lipids may be a promising target to explore in more detail as the typical structural organization of dietary lipids in human milk, *i.e.* being present as large lipid droplets enveloped by the MFGM, may contribute to some of these benefits. Mimicking the supramolecular structure of lipid droplets in human milk more closely could further improve IMF lipid quality and support brain development of formula fed or mixed fed infants specifically.

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